Bioactivation of aflatoxin B1 in the nasal and tracheal mucosa in swine

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Bioactivation of Aflatoxin B_1 in the Nasal and Tracheal Mucosa in Swine[1]

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ABSTRACT: Whole-body autoradiography of ^{3}H-labeled aflatoxin B_1 in young pigs showed a localization of bound label in the nasal olfactory and respiratory mucosa, in the tracheo-laryngeal mucosa, and in the conjunctiva, in addition to the liver. Whole-body and microautoradiography also showed a labeling of pigmented tissues, which can be ascribed to a melanin binding of AFB_1. In vitro experiments with microsomal preparations of various tissues from sows revealed that the nasal respiratory and olfactory mucosa had the highest capacity to form DNA-bound aflatoxin B_1-metabolites. The tracheal mucosa and the liver, in order, had lesser binding capacity. The lung was found to be devoid of aflatoxin B_1-bioactivating capacity. In vitro microautoradiography revealed bound label in specific cell types in the nose and trachea and in some cells of the conjunctiva. A drastic decrease in the aflatoxin B_1-DNA binding was observed when microsomal preparations of the nasal respiratory and olfactory mucosa were incubated in the presence of reduced glutathione, but without any addition of cytosolic glutathione-*S*-transferases. In incubations of liver microsomes under these conditions a somewhat lower inhibition of the aflatoxin B_1-DNA binding was seen. Our results demonstrate that the nasal olfactory and respiratory mucosa and the tracheal mucosa have a higher capacity than the liver to bioactivate aflatoxin B_1 in swine. Our data further show that microsomal-associated glutathione-*S*-transferases with a high capacity to catalyze the conjugation of the reactive aflatoxin B_1-epoxide to reduced glutathione are present in the nasal olfactory and respiratory mucosa of swine.

Key Words: Aflatoxins, Pigs, Conjunctiva, Melanins, Glutathione Transferase

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Introduction

Aflatoxin B_1 (**AFB_1**) is a mycotoxin produced by the fungi *Aspergillus flavus* and *Aspergillus parasiticus*. Farm animals can be exposed to AFB_1 via ingestion or inhalation of moldy feed or feed dust. Among domestic livestock, swine are reported to be the most sensitive species to aflatoxin intoxication (Diekman and Green, 1992). Clinical signs of aflatoxicosis in swine are rather nonspecific and include loss of appetite, diarrhea, coughing, immunosuppression, and impaired reproductive efficiency (Cook et al., 1989; van Heugten et al., 1994). Necroscopy findings include swollen and yellow liver with centrilobular necroses (Newberne and Butler, 1969; Cook et al., 1989).

Aflatoxin B_1 is relatively nontoxic per se but is bioactivated by cytochrome P450 enzymes to a reactive intermediate, the AFB_1-8,9-epoxide. Several forms of P450 isoenzymes are known to catalyze the bioactivation (Aoyama et al., 1990; Crespi et al., 1991; Eaton and Gallhagher, 1994). The reactive intermediate binds to nucleic acids and other macromolecules, thereby inducing the toxic and carcinogenic effects (Essigmann et al., 1982). The AFB_1-epoxide may also be detoxified by conjugation with reduced glutathione (**GSH**) via the action of glutathione-*S*-transferases (**GST**) (Quinn et al., 1990).

There are to our knowledge no reports dealing with the bioactivation or GSH conjugation of AFB_1 in swine. In this study we have examined the disposition of ^{3}H-labeled AFB_1 (**^{3}H-AFB_1**) in swine. The aim was to trace tissues with a capacity to bioactivate AFB_1 and to evaluate the detoxification of the AFB_1-8,9-epoxide by GSH conjugation in these tissues.

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Materials and Methods

Chemicals. [G-^{3}H]AFB_1 with a specific radioactivity of 28 Ci/mmol was purchased from Moravek Biochemicals (Brea, CA). The ^{3}H-AFB_1 was purified by liquid chromatography before use by a method described previously (Larsson et al., 1989). Non-labeled AFB_1, calf thymus DNA, GSH and glucose-6-phosphate dehydrogenase were purchased from Sigma Chemical (St. Louis, MO). Other chemicals used in the study were of analytical grade and obtained from regular commercial sources.

Animals. Five healthy sows of a crossbreed of Yorkshire/Hampshire/Swedish Landrace (Piggham) maintained on adequate diet with no history of previous exposure to AFB_1 were used for the in vitro experiments. The sows were slaughtered at a local abattoir. Tissues to be examined were taken immediately after death, placed in ice-cold .9% saline, and transported to the laboratory for further preparation. For whole-body autoradiography and in vivo microautoradiography two 10-d-old suckling pigs of the same breed were used.

Whole-Body Autoradiography. Two pigs were injected into an abdominal vein with ^{3}H-AFB_1 (.22 mCi, 2.5 μg AFB_1/kg BW) and killed by an overdose of pentobarbital sodium (60 mg/kg BW i.v.) after 20 min and 4 h, respectively. The animals were immediately frozen, embedded in carboxymethyl cellulose, and sectioned for autoradiography, according to the method of Ullberg et al. (1982). To study the firmly bound radioactivity every other freeze-dried tissue section was extracted successively with 5% trichloroacetic acid, 50% ethanol, 99.5% ethanol, and heptane for 1 min, and then rinsed with tap water for 5 min. The sections were dried and pressed against x-ray film (Hyperfilm-^{3}H, Amerham INT. plc, Little Chalfont, U.K.) together with adjacent non-extracted sections.

Microautoradiography. In vivo microautoradiography was performed with skin pieces taken from the abdomen and the ear of the pigs used for the whole-body autoradiography. The skin pieces were removed and fixed in 4% formaldehyde in phosphate buffer, pH 7.4, before the pigs were used for the whole-body autoradiography.

In vitro microautoradiography was performed with nasal olfactory and respiratory mucosa, tracheal mucosa, and conjunctiva. The tissues were removed from a sow and incubated with 2 μCi of ^{3}H-AFB_1 (.16 μM) in 2 mL of 50 m*M* Tris-HCl buffer, pH 7.4, containing 5 m*M* glucose-6-phosphate. The incubations were performed for 1 h at 37°C in O_2 atmosphere. After the incubations the pieces were fixed in the phosphate-buffered formalin.

The tissues from the in vivo and in vitro experiments were dehydrated in an ethanol serie and embedded in Technovit 100 (Heraeus Kulzer GmbH, Wehrheim, Germany). Sections (2 μm thick) were cut on glass slides, covered with Kodak NTB-2 emulsion, and stained according to the periodic acid-Schiff technique (PAS) or with toluidine blue as described previously (Larsson et al., 1990). Exposure was for 3 wk to 4 mo at 4°C, followed by photographic development and fixation. The extensive extractions during the fixation and embedding procedures will remove all unbound radioactivity, and the autoradiograms will therefore show only tissue-bound radioactivity (Larsson and Tjälve, 1992).

Preparation of Subcellular Fractions. Microsomes of sow tissues were prepared from the nasal olfactory, nasal respiratory and tracheal mucosa, and the liver and lung. The tissues were homogenized in 50 m*M* Tris-HCl buffer, pH 7.4, containing .15 *M* KCl. The homogenates were sedimented at 10,000 × *g* for 25 min. To obtain microsomes the resulting supernatants were centrifuged at 105,000 × *g* for 1 h. The microsomal pellets were washed in the Tris-HCl buffer and recentrifuged at 105,000 × *g* for another hour before resuspension in the same buffer and storage at –70°C until used. Protein content of microsomes was determined according to the method of Lowry et al. (1951).

In Vitro Biotransformation of AFB_1. Incubations were performed at 37°C for 20 min in a total volume of 2 mL of O_2-saturated 50 m*M* Tris-HCl buffer, pH 7.4, containing .8 m*M* NADP, 5 m*M* glucose-6-phosphate, .4 U glucose-6-phosphate dehydrogenase/mL, 3 m*M* $MgCl_2$, 50 m*M* KCl, .5 mg of calf thymus DNA, and .5 μCi ^{3}H-AFB_1 (.16 μM AFB_1) dissolved in 1 μL of methanol. Microsomal preparations of the tissues mentioned above, with a protein content corresponding to .1 or .2 mg, were added to the incubations. In some incubations 5 m*M* GSH was added to the incubation media. Incubations kept on ice served as controls. The incubations were stopped by adding .5 mL of 4.5 *M* NaCl and .1 mL of 3% sodium dodecylsulphate. The amount of ^{3}H-AFB_1 metabolites bound to DNA was determined by extractions and liquid scintillation counting as described earlier (Tjälve et al., 1992).

Results

Whole-Body Autoradiography. Whole-body autoradiography of the freeze-dried tissue sections of the pig killed 20 min after the i.v. injection of ^{3}H-AFB_1 showed a homogeneous distribution of radioactivity in the blood and most of the tissues in the body. Radioactivity that markedly exceeded the level in the blood was found in the liver parenchyma, the nasal olfactory and respiratory mucosa, and the tracheal and laryngeal mucosa (Figures 1a and 2a). There was also a considerable labeling of pigmented tissues, such as the melanin in the eyes and hair follicles and in the palpebral and bulbar conjunctiva (Figure 3a). In addition, there was a high labeling in the kidney and in the contents of the urinary bladder

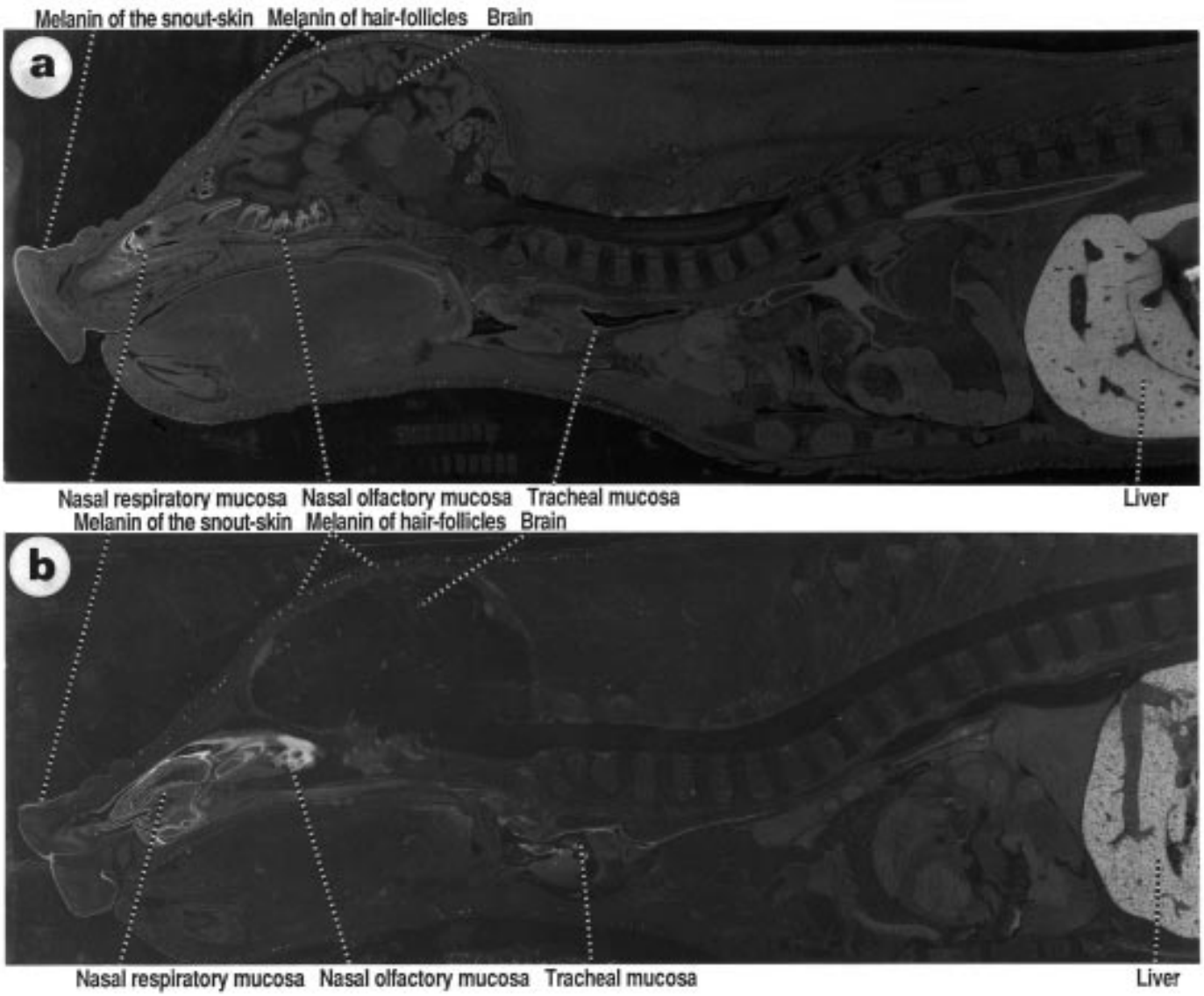

Figure 1. Whole-body autoradiograms of freeze-dried sections of young pigs killed (a) 20 min or (b) 4 h after i.v. injections of ^{3}H-AFB$_1$ (2.5 μg/kg BW).

and small intestine. In the central nervous system the radioactivity in the gray matter somewhat exceeded the level in the blood, whereas the label of the white matter was low (Figure 1a). Whole-body autoradiography of the extracted tissue sections of this pig showed retention of radioactivity in only a few tissues. Thus, a marked tissue-bound label was found in the liver parenchyma, the nasal olfactory and respiratory mucosa, and the melanin-containing tissues (Figure 2b). A somewhat lower level of firmly bound radioactivity was found in the tracheal and laryngeal mucosa and in the conjunctiva. The bound label in the liver showed an irregular pattern, the highest level probably being present in the centrilobular parts.

In whole-body autoradiograms of freeze-dried sections of the pig killed 4 h after the i.v. injection of ^{3}H-AFB$_1$, the distribution picture was similar to the one seen in the pig killed after 20 min (Figure 1b). However, there were some distinct differences. In the pig killed at 4 h the labeling of the gray matter in the central nervous system had disappeared. The evenly distributed label of most tissues of the body was also much lower in the pig killed at 4 h compared with the one killed after 20 min (Figure 1b). The label in the liver showed an irregular pattern, the highest level probably being present in the centrilobular parts (Figure 3b). In the autoradiograms of extracted tissue sections from the pig killed at 4 h the tissues retaining bound label were similar to those in the pig with the shorter survival interval. However, the firmly bound radioactivity in the tracheal and laryngeal mucosa was more pronounced in the pig killed at 4 h.

Microautoradiography. The in vivo microautoradiography showed a localization of bound label over the

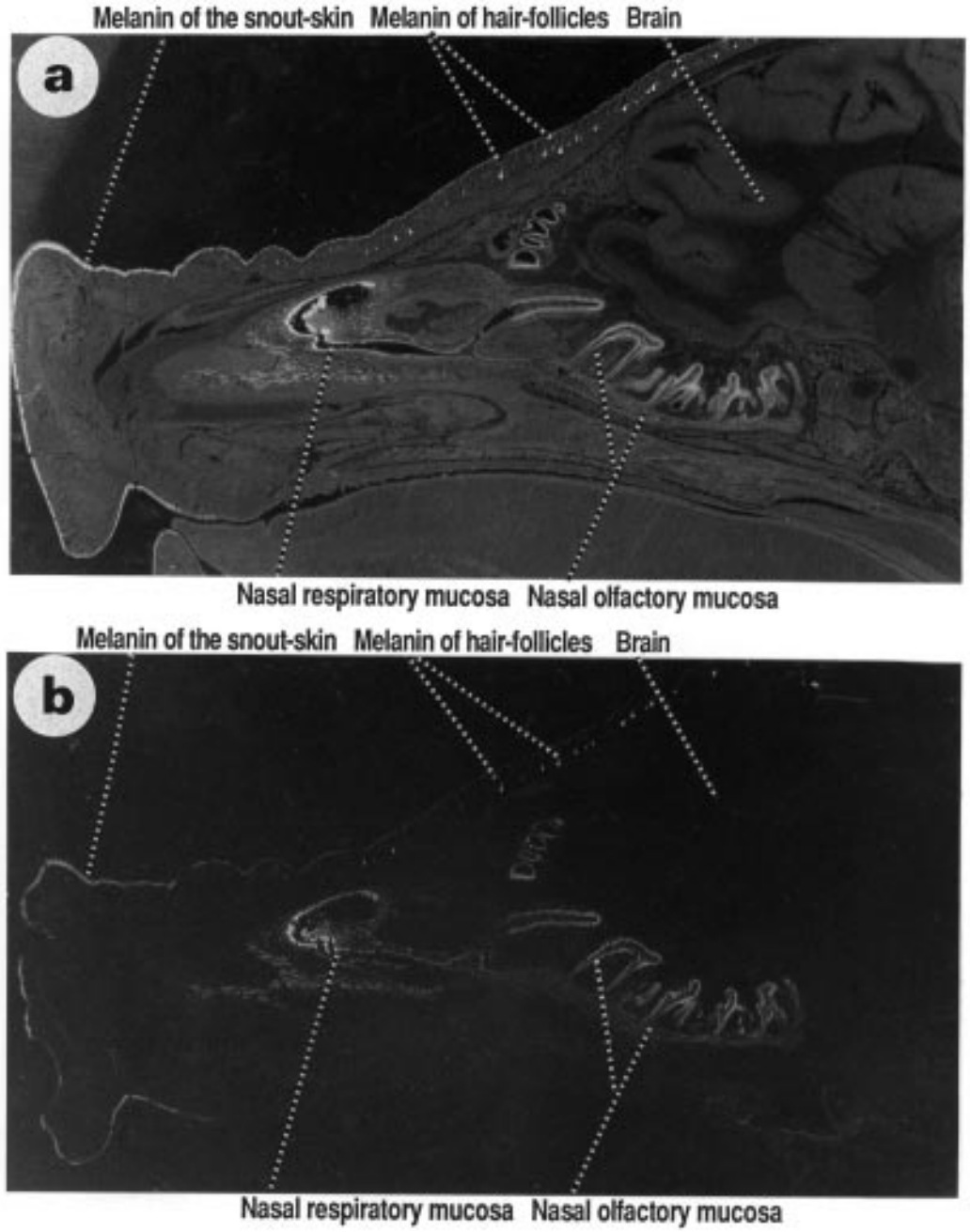

Figure 2. Details of whole-body autoradiograms of a freeze-dried section (a) and an adjacent extracted section (b) of a young pig killed 20 min after an i.v. injection of ^{3}H-AFB_1 (2.5 μg/kg BW). The autoradiograms show the localization of the total radioactivity (a) and the tissue-bound radioactivity (b) in the head of the pig.

pigment granules in the epidermal melanocytes and in the hair follicles (Figures 4a and b).

In the in vitro microautoradiography of the nasal olfactory mucosa there was a localization of silver grains over the cells of Bowman's glands in the lamina propria mucosae (Figure 5a). In vitro autoradiography of respiratory epithelium showed label only over acini of some serous glands, whereas in the mucous glands and other structures no label exceeding the background level was seen (Figure 5b). In the tracheal mucosa, silver grains were localized preferentially over the mucous cells (Figure 5c). The in vitro microautoradiography of the conjunctiva showed silver grains in cells present in the middle and superficial parts of the epithelium (Figure 5d). In all tissues examined there was a preferential localization of the silver grains over the nuclei of the cells in the labeled structures.

Formation of DNA-Bound AFB_1 Metabolites by Microsomes from Various Tissues. Incubations with microsomes demonstrated that among the tissues examined, the nasal respiratory and the olfactory

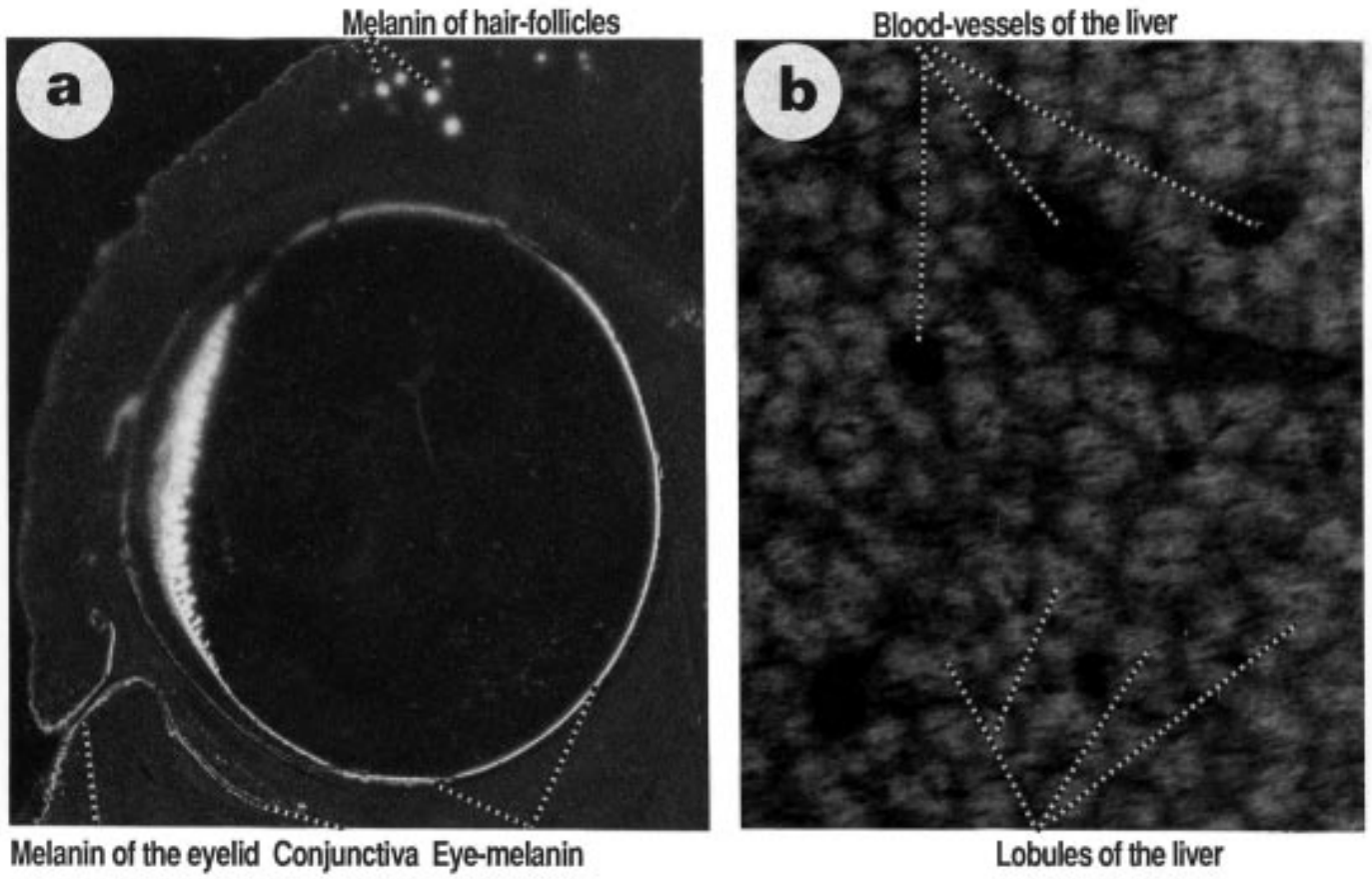

Figure 3. Details of whole-body autoradiograms of freeze-dried tissue sections of young pigs killed (a) 20 min or (b) 4 h after i.v. injections of ^{3}H-AFB_1 (2.5 μg/kg BW). The autoradiograms show the labelling of the eye region (a) and of the liver (b).

mucosa had the highest capacity to form DNA-bound AFB_1 metabolites (Table 1). The respiratory mucosa was somewhat more efficient than the olfactory mucosa in this respect. Thereafter followed in decreasing order the tracheal mucosa and the liver. The lung was found to be devoid of AFB_1-bioactivating capacity.

Effects of GSH on the DNA-binding of ABF_1. The presence of GSH in the incubations with microsomal preparations from the nasal olfactory and respiratory mucosa potently inhibited the binding of AFB_1 to DNA (Table 2). In the incubations with liver microsomes in the presence of GSH there was also a substantial inhibition of the AFB_1-DNA binding. However, the relative inhibition was lower than in the nasal respiratory and olfactory mucosa.

Discussion

The present study has shown that some extrahepatic tissues have a high capacity to bioactivate AFB_1 in swine. Our results demonstrate that the accumulation of firmly bound metabolites in the tissues in vivo correlates with a capacity to bioactivate AFB_1 to DNA-bound metabolites in vitro. These data confirm that the bioactivation of AFB_1 occurs locally in the extrahepatic tissues.

The in vitro experiments showed that the mucosal linings of the olfactory and respiratory parts of the nose had the highest AFB_1-bioactivating capacity. This may be explained by the presence of P450 isoenzymes with a high capacity to form the AFB_1-epoxide in these tissues. It has also been reported that there is a high ratio of NADPH cytochrome *c* reductase:cytochrome P450 in the nasal olfactory mucosa (Reed et al., 1986; Larsson et al., 1989). This property may facilitate the microsomal electron transport to cytochrome P450 and consequently promote the bioactivation of AFB_1.

In addition to the nasal mucosa, AFB_1-bioactivation was demonstrated in the tracheal and laryngeal mucosa and in the conjunctival mucosa in swine. We have previously shown that several extrahepatic tissues are able to bioactivate AFB_1 in cows, sheep, mice, rats, and monkeys (Larsson et al., 1989; Larsson et al., 1990; Tjälve et al., 1992; Larsson et al., 1994; Larsson and Tjälve, 1992, 1993, 1995). In all species, the nasal mucosa was found to be the most

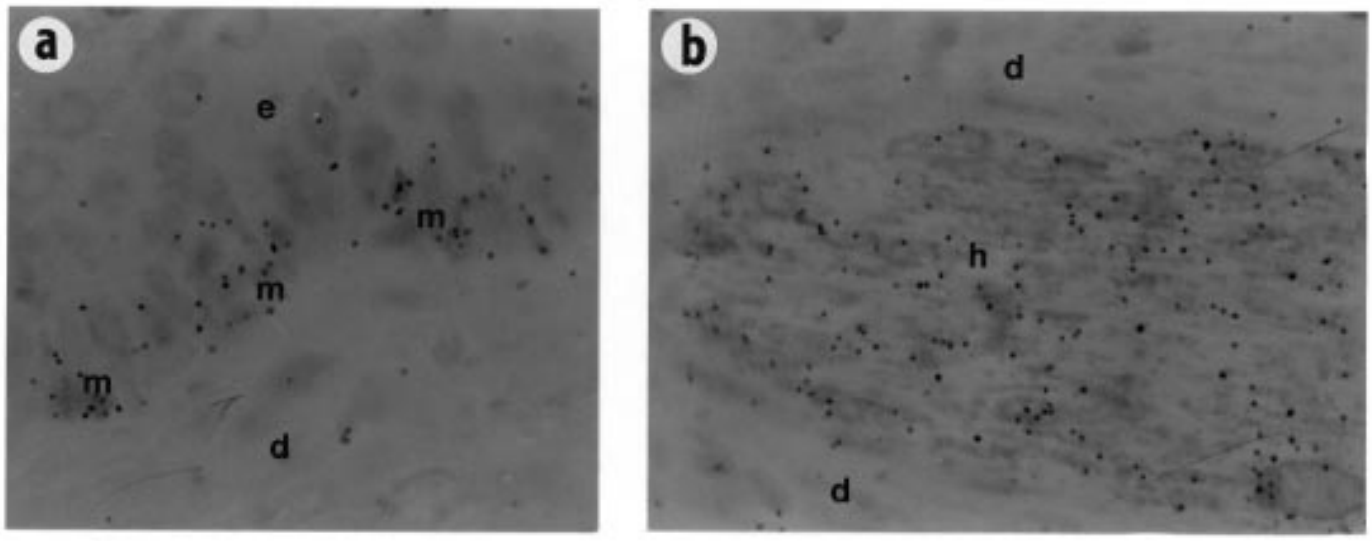

Figure 4. Microautoradiograms of skin-pieces from a young pig killed 20 min after an i.v. injection of ^{3}H-AFB_1 (2.5 μg/kg BW). (a) shows silver grains over melanocytes at the junction between the dermis and the epidermis. (b) shows silver grains over pigment granules in a hair-follicle. d, dermis; e, epidermis; h, hair follicle; m, melanocytes (toluidine blue; ×670).

potent tissue in this respect. We have shown bioactivation of AFB_1 in the tracheal mucosa in sheep and rats (Larsson et al., 1994; Larsson and Tjälve, 1995) and AFB_1-bioactivation in this tissue has also been shown in monkeys, rabbits, and hamsters (Wilson et al., 1990). In contrast to swine the oesophageal mucosa and Clara cells in the bronchioles have been found to bioactivate AFB_1 in mice, monkeys, and sheep (Larsson and Tjälve, 1992, 1993; Larsson et al., 1994). Our observation that the conjunctiva seems to have an ability to bioactivate AFB_1 in swine coincides with previous results in sheep (Larsson et al., 1994). It seems that the spectrum of extrahepatic tissues engaged in the bioactivation of AFB_1 correlates in most aspects in the various species, although in some instances marked species differences exist.

The in vitro microautoradiography showed that the bioactivation of AFB_1 in the extrahepatic tissues is confined to a few cell types, such as the cells of Bowman's glands in the nasal olfactory epithelium, cells of serous glands in the nasal respiratory epithelium, and the mucous cells in the tracheal mucosa. These cell types are known to contain P450 enzymes (Plopper et al., 1987; Dahl and Hadley, 1991) that are needed to bioactivate AFB_1. The localization of silver grains over the nuclei of the labeled cells has previously been observed in extrahepatic tissues of other species in studies with AFB_1

Table 1. Formation of DNA-bound AFB_1-metabolites by microsomal preparations of tissues from swine[a]

Tissue	Amount of microsomal protein added, mg	
	.1	.2
	— pmol AFB_1-metabolites bound/mg of DNA[b] —	
Nasal respiratory mucosa	7.2 ± .3	19.4 ± .2
Nasal olfactory mucosa	4.1 ± .2	15.4 ± 1.8
Tracheal mucosa	1.7 ± .1	4.4 ± .1
Liver	.5 ± .1	1.0 ± .1
Lung	.1 ± .1	.2 ± .1
Control[c]	NE[d]	.1 ± .1

[a]Incubations were carried out for 15 min with .5 mg of DNA, .45 μCi ^{3}H-AFB_1 (.16 μM), and .1 or .2 mg of microsomal protein.
[b]Values are expressed as mean ± SE; n = 3.
[c]Incubations on ice served as controls.
[d]NE = not examined.

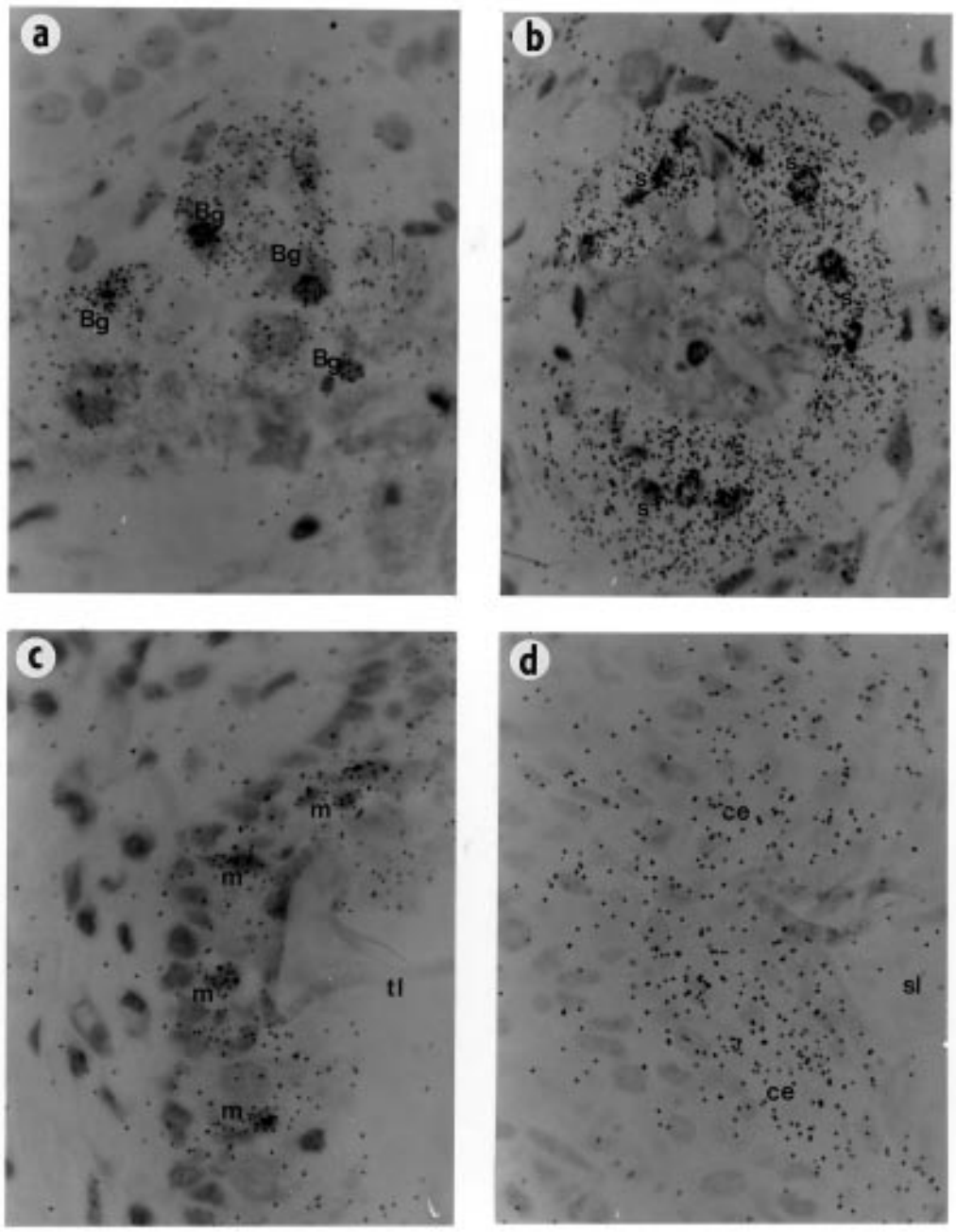

Figure 5. Microautoradiograms of the (a) nasal olfactory mucosa, (b) nasal respiratory mucosa, (c) trachea, and (d) conjunctiva. Swine tissues were incubated with ^{3}H-AFB$_1$ (.16 μM) for 1 h in a buffer under O_2 atmosphere. Bg, cells of Bowman's glands in the olfactory lamina propria mucosa; ce, conjunctival epithelium; m, mucous cells of the tracheal mucosa; s, cells of a serous gland in the nasal respiratory epithelium; sl, slit between the conjunctiva and the cornea; tl, tracheal lumen. Note the preferential labeling of the nuclei in some cells in the tissues (toluidine blue; ×525).

(Larsson et al., 1989; Larsson and Tjälve, 1992). It has been shown that AFB$_1$-epoxide binds readily to double-stranded DNA (Yu et al., 1990) and the labeling of the nuclei may reflect a specific affinity of the bioactivated AFB$_1$ for the DNA.

The autoradiography showed a retention of labeled material in the melanin-containing tissues of the eye and the skin. We have previously reported an accumulation of AFB$_1$ in pigmented tissues in other species (Larsson et al., 1988, 1994), which was determined to be related to a melanin-affinity of non-metabolized AFB$_1$ (Larsson et al., 1988). The biological implication of the melanin-binding of AFB$_1$ is not known. The melanin may function as a protective filter by binding

Table 2. Effects of GSH (5 m*M*) on the formation of DNA-bound AFB_1-metabolites by microsomal preparations of tissues from swine[a]

Tissue	pmol AFB_1-metabolites bound/mg of DNA[b]		
	Without GSH (A)	With GSH (B)	B:A, %
Nasal respiratory mucosa	15.9 ± 1.0	.5 ± .1	3
Nasal olfactory mucosa	14.3 ± .4	.9 ± .1	6
Liver	1.2 ± .02	.4 ± .02	33

[a]Incubations were performed for 15 min with .2 mg of microsomal protein, 1 mg of DNA, and .45 μCi ^{3}H-AFB_1 (.16 μM).
[b]Values are expressed as mean ± SE; n = 3.

potentially harmful substances such as AFB_1 and thus withdrawing them from critical target sites (for review, see Larsson, 1993). On the other hand, the binding of AFB_1 is reversible (Larsson et al., 1988), which will result in a release of AFB_1 into the cytoplasm of the melanin-containing cells, and possibly into surrounding cells. The possibility that the substance may induce malignant melanoma should therefore be considered. Melanotic tumors have been observed in swine (Pulley and Stannard, 1990), but the potential role of AFB_1 in the development of these neoplasms have not been explored.

The incubations with microsomal preparations of various tissues in the presence of GSH indicate that swine have microsomal associated GST with a high capacity to catalyze the GSH-conjugation of the AFB_1-epoxide. We have previously shown that several tissues of sheep contain microsomal-associated GST that are capable of catalyzing the AFB_1-epoxide GSH-conjugation (Larsson et al., 1994). However, studies in other species have shown that the conjugation reaction between the AFB_1-epoxide and GSH is catalyzed mainly by cytosolic GST (Lotlikar et al., 1980; Tjälve et al., 1992). Further studies are needed to explore the role of the microsomal-associated GST in the detoxification of AFB_1.

The liver is a target for the AFB_1 toxicity in swine, as well as in other species. Our autoradiographic observation that the bound AFB_1 radioactivity in the liver was localized to the central parts of the liver lobuli correlates with reports of centrilobular necrosis of the liver in aflatoxicosis in swine (Newberne and Butler, 1969). Aflatoxin B_1 induces liver tumors in experimental animals, and in some areas of the world liver cancer in humans has been correlated with high levels of aflatoxin contamination (Busby and Wogan, 1984). Hepatocellular carcinoma has been observed in swine (Popp, 1990), but it is not known whether AFB_1 is a causative factor. Also, the extrahepatic tissues with a capacity to bioactivate AFB_1 are potential targets for the toxic and carcinogenic effects of the substance. Ethmoidal carcinomas of the nasal cavity have been observed in swine exposed to AFB_1 via heavily contaminated feed (Rajan et al., 1972) and a role of the mycotoxin in the aetiology of this neoplasm seems possible. Ethmoidal carcinomas have been observed in cattle and sheep in some countries (Yonemichi et al., 1978; Sreekumaran and Rajan, 1983). In sheep similar tumors have been observed in animals experimentally exposed to AFB_1 (Lewis et al., 1967). It is thus possible that AFB_1 may be an etiological factor in the development of nasal tumors in several species. It has been shown that AFB_1 can be present in high concentrations in respirable grain dust particles (Sörenson et al., 1981; Burg and Sothwell, 1984). This may lead to a local exposure of the nasal mucosa to AFB_1, which conceivably may increase the risk for tumorigenesis of this tissue. Presumably AFB_1 may reach the nasal mucosa both via contaminated inhaled respiratory dust particles and via the blood following absorption from the gastrointestinal tract. It is not known whether the trachea may be a target for negative effects of AFB_1 in swine. Tracheal tumors have been reported to be extremely rare in domestic animals (Moulton, 1990). However, tracheal instillation of AFB_1 in rats has been shown to result in neoplasms of this tissue (Dickens et al., 1966). As mentioned, AFB_1 bioactivation occurs in the tracheal mucosa in rats (Larsson and Tjälve, 1995), and local exposure of such a tissue may promote carcinogenesis.

Implications

The extrahepatic tissues with a capacity to bioactivate aflatoxin B_1 may be sites of toxic and carcinogenic effects of the substance. In swine the nasal olfactory and respiratory mucosa and the tracheolaryngeal mucosa are such potential targets. Additional tissues that in swine may be sensitive to aflatoxin B_1 toxicity are the pigmented tissues and the conjunctiva. Feed dust particles may be contaminated with aflatoxin B_1 and a local exposure of the tissues, related to this contamination, may promote the toxicity and carcinogenicity.

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